AD_____

Award Number: DAMD17-00-1-0629

TITLE: Cyclin E as a Prognosticator for Node Negative, Stage II Breast Cancer

PRINCIPAL INVESTIGATOR: Khandon Keyomarsi, Ph.D.

CONTRACTING ORGANIZATION: The University of Texas M.D. Anderson Cancer Center
Houston, Texas 77030

REPORT DATE: October 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2001	Final (1 Sep 00 - 31 Aug 01)

4. TITLE AND SUBTITLE
Cyclin E as a Prognosticator for Node Negative, Stage II Breast Cancers

5. FUNDING NUMBERS
DAMD17-00-1-0629

6. AUTHOR(S)
Khandon Keyomarsi, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Texas M.D. Anderson Cancer Center
Houston, Texas 77030

E-Mail: kkeyomar@mail.mdanderson.org

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Our laboratory has made the novel observation that cyclin E is overexpressed and present in lower molecular weight (LMW) isoforms in selected breast cancer cells and tumor tissues. In a retrospective analysis of tumor specimens from 403 breast cancer patients, we previously discovered that overexpression of LMW isoforms cyclin E strongly correlated with the development of distant metastases and poor overall survival. With a median follow-up of 4-years 91.7% of breast cancer cases with cyclin E overexpression developed distant metastases compared to 7% of cases without cyclin E overexpression ($p < 0.001$). We also found that in 126 patients with Stage I breast cancer, cyclin E overexpression had a 100% predictive value for the development of metastatic disease. Based on this retrospective work, our goal for this award was to initiate a PROSPECTIVE study assessing the importance of cyclin E overexpression as a negative predictor of outcome in Stage I and II breast cancers. We have been able to accrue over 120 diagnosed with Stage I and II disease, obtaned normal and tumor tissue from these patients and subjected them to western blot analysis with cyclin E antibody. We will do follow-up on these patients for the next 3-5 years and correlate the expression of cyclin E to outcome at the end of the follow-up period.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Cell cycle, G1 cyclins, prognostic marker, anatomical samples	10
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

TABLE OF CONTENTS

Introduction:

The overall purpose of this 1 year study was to initiate a prospective study assessing the ability of cyclin E as a predictor of poor outcome only in stage I and II node negative breast cancers.

Body:

Despite significant recent advances in understanding the cell biology and molecular genetics of breast cancer, patient prognosis continues to be primarily determined by the pathological extent of disease in the axillary lymph nodes (1). However, approximately 1/3 of women with lymph node-negative breast cancer who do not receive systemic treatment develop disease recurrence while approximately 1/3 of patients with positive lymph nodes will be free of recurrence 10 years after local-regional therapy alone (2, 3). These data highlight the need for more sensitive and specific prognostic factors. Such factors would help to more precisely inform patients and physicians about expected outcomes and allow for better individualization of systemic treatment use.

A number of biological factors have been studied over the past two decades in an attempt to further refine risk categories of breast cancer. Our efforts to find important biological markers in breast cancer have focused on the G1/S checkpoint and how deregulation of this critical phase of the cell cycle affects the virulence and metastatic potential of breast cancer (4-8). We have specifically been interested in cyclin E because of its importance in the regulation of G1 to S transition in normal dividing cells (9, 10). In addition, abnormal overexpression of the cyclin E protein can result in acceleration through G1 (11, 12).

Cyclin E gene is amplified in some breast cancer cell lines (6, 13) and we have shown that this amplification can result in as much as 64-fold overexpression of cyclin E mRNA that is constitutively expressed across all phases of the cell cycle (8, 14). Other investigators have documented a strong correlation between cyclin E overexpression and lack of estrogen receptor expression (5, 15, 16). More important, we and others have found that high cyclin E levels were associated with a significantly increased risk of death and/or relapse from breast cancer (7, 16, 17), although some studies did not show an association (17, 18).

We discovered that some breast cancer cell lines and human breast cancers can express up to 5 lower molecular weight (LMW) isoforms of cyclin E (ranging in size from 34 to 49 kDa), in addition to overexpression of the full-length cyclin E 50 kDa protein (6, 7, 16, 19). We hypothesized that these LMW isoforms are active and that the expression of these isoforms correlates with the stage and prognosis of breast cancer (7, 16, 20, 21). The previous studies that have examined the prognostic importance of cyclin E in breast cancer have used immunohistochemistry (IHC) staining to detect alteration in cyclin E expression (17, 18). The antibodies used in these studies cannot distinguish between the form of cyclin E found in both normal and tumor cells and those forms of the protein that are unique to tumor cells.

We performed a RETROSPECTIVE study to test our hypothesis and to more fully evaluate the clinical importance of LMW forms of cyclin E in breast cancer. For these retrospective analysis we examined tumor specimens from 403 breast cancer patients and observed that A) Cyclin E is a superior and independent prognostic marker for breast cancer as compared to other tumor and cell cycle markers (i.e.: age, estrogen and progesterone receptor status, DNA index, ploidy, proliferation index, lymph node involvement, Her-2, cyclin D1, p21, p27, p16 and pRb). These analyses revealed that breast cancers that overexpress cyclin E have a

91.7% probability ($p < 0.001$) of having poor prognosis over a 48-month average follow up period. B) In addition, we found that cyclin E overexpression in Stage I breast cancer has a 100% predictive value to poor prognosis in our patient population; i.e. twelve patients with Stage I disease (from 126 Stage I patients) overexpressed cyclin E- all 12 patients expired of disease. Cyclin E was NOT altered in any of the remaining 114 Stage I patients. Collectively the results from our retrospective study revealed that cyclin E and its LMW isoforms proved to be the most powerful independent predictor for survival in stage I-III breast cancer.

Based on these findings our one year goal is to initiate a PROSPECTIVE study assessing the ability of cyclin E as a predictor of poor outcome only in Stage I and II node negative breast cancers. We will also examine the activity and immune-complex formation of cyclin E in extracts prepared from freshly resected tumors. Our long term goal is that once patients with deregulated cyclin E expression are identified, they may then be singled out for therapy that is more aggressive than what currently administered to node negative Stage I or II patients. Specifically, our objectives were to

1: To use cyclin E antibody as a prognostic marker for stage I and II breast cancer in a PROSPECTIVE study.

2: Examine the cyclin E associated activity and its immune-complex formation with key cell cycle regulators in freshly resected tumor samples.

Results/Key Research Accomplishments:

During the past year we were successful in initiating our prospective analysis.

1. We obtained freshly resected breast tissue samples (50 mg each of normal adjacent and tumor) from 130 patients diagnosed with stage I and Stage II node negative breast tumors were collected and protein were extracted from each sample.

2. Using monoclonal as well as a polyclonal (generated in my laboratory) antibodies to cyclin E we analyzed the alteration of cyclin E on freshly resected tumors by Western blot analysis.
3. We correlated the cyclin E alterations in samples from (b) with the expression of currently used markers such as the Her-2/neu, EGFR, PCNA, p53, p27, and cyclin D1.
4. Protein extracted from freshly resected breast tissue samples (from 1) was subjected to cyclin E associated kinase activity using polyclonal antibodies to cyclin E in immunoprecipitate (IP) assays using Histone H1 and GST-Rb as substrates.
5. Immune-complexes formed between cyclin E, CDK2, p21, and p27 has been assessed by first IPing tumor tissue extracts with cyclin E antibody, followed by Western blot analysis with the aforementioned antibodies.

At the end of this initial year of our study it is premature to know whether the overexpression and increased activity of cyclin E are predictors of poor outcome in breast cancer patients since these patients have to be followed for 3-5 years, post diagnosis. However, we have been able to make some initial conclusions about the study at this point

1. The overexpression of cyclin E was observed in 18-20% of all cases examined. This percent overexpression recapitulates our retrospective analysis.
2. We observed an inverse correlation between overexpression of cyclin E and low expression of p27, indicative of poor prognosis. Reciprocally, in all specimens with low expression of cyclin E, p27 was overexpressed. These observations also recapitulate what is known about the contribution of these 2 factors to predicting poor outcome.
3. The cyclin E overexpression is independent of the node status of the patients-again, recapitulating our retrospective results.

Conclusion/Reportable Outcome:

During the one-year study, we have accomplished the goals set forth by our proposal. We have accrued the desired number of patients and analyzed them for the biomarkers proposed. Our initial analysis reveals that the overexpression of cyclin E occurs in the same number of patients as those observed in our retrospective analysis. We are now in a position to examine the role of such cyclin E overexpression with prognosis.

References

1. Voogd, A. C., Nielsen, M., Peterse, J. L., Blichert-Toft, M., Bartelink, H., Overgaard, M., van Tienhoven, G., Andersen, K. W., Sylvester, R. J., and van Dongen, J. A. Differences in risk factors for local and distant recurrence after breast-conserving therapy or mastectomy for stage I and II breast cancer: pooled results of two large European randomized trials, J Clin Oncol. *19:* 1688-97., 2001.
2. Polychemotherapy for early breast cancer: an overview of the randomised trials. Early Breast Cancer Trialists' Collaborative Group, Lancet. *352:* 930-42., 1998.
3. Tamoxifen for early breast cancer: an overview of the randomised trials. Early Breast Cancer Trialists' Collaborative Group, Lancet. *351:* 1451-67., 1998.
4. Dou, Q.-P., Pardee, A. B., and Keyomarsi, K. Cyclin E- a better prognostic marker for breast cancer than cyclin D?, Nat Med. *2:* 254, 1996.
5. Gray-Bablin, J., Zalvide, J., Fox, M. P., Knickerbocker, C. J., DeCaprio, J. A., and Keyomarsi, K. Cyclin E, a redundant cyclin in breast cancer, Proc Natl Acad Sci USA. *93:* 15215-15220, 1996.

6. Keyomarsi, K. and Pardee, A. B. Redundant cyclin overexpression and gene amplification in breast cancer cells, Proc Natl Acad Sci USA. *90:* 1112-1116, 1993.

7. Keyomarsi, K., O'Leary, N., Molnar, G., Lees, E., Fingert, H. J., and Pardee, A. B. Cyclin E, a Potential Prognostic Marker for Breast Cancer, Cancer Res. *54:* 380-385, 1994.

8. Keyomarsi, K., Conte, D., Toyofuku, W., and Fox, M. P. Deregulation of cyclin E in breast cancer, Oncogene. *11:* 941-950, 1995.

9. Lew, D. J., Dulic, V., and Reed, S. I. Isolation of three novel human cyclins by rescue of G1 cyclin (cln) function in yeast, Cell. *66:* 1197-1206, 1991.

10. Dou, Q.-P., Levin, A. H., Zhao, S., and Pardee, A. B. Cyclin E and cyclin A as candidates for the restriction point protein, Cancer Res. *53:* 1493-1497, 1993.

11. Ohtsubo, M., Theodoras, A. M., Schumacher, J., Roberts, J. M., and Pagano, M. Human cyclin E, a nuclear protein essential for the G1-to-S phase transition, Mol Cell Biol. *15:* 2612-2624, 1995.

12. Resnitzky, D., Gossen, M., Bujard, H., and Reed, S. I. Acceleration of the G1/S phase transition by expression of cyclins D1 and E with an inducible system, Mol Cell Biol. *14:* 1669-1679, 1994.

13. Buckley, M. F., Sweeney, K. J. E., Hamilton, J. A., Sini, R. L., Manning, D. L., Nicholson, R. I., deFazio, A., Watts, C. K. W., Musgrove, E. A., and Sutherland, R. L. Expression and amplification of cyclin genes in human breast cancer, Oncogene. *8:* 2127-2133, 1993.

14. Sgambato, A., Doki, Y., Schieren, I., and Weinstein, I. B. Effects of cyclin E overexpression on cell growth and response to transforming growth factor ß depend on cell context and p27kip1 expression, Cell Growth Diff. *8:* 393-405, 1997.

15. Scott, K. and Walker, R. Lack of cyclin E immunoreactivity in non-malignant breast and association with proliferation in breast cancer, Br J Cancer. *76:* 1288-1292, 1997.

16. Nielsen, N. H., Arnerlov, C., Emdin, S. O., and Landberg, G. Cyclin E overexpression, a negative prognostic factor in breast cancer with strong correlation to oestrogen receptor status, Br J Canc. *74:* 874-880, 1996.

17. Porter, P. L., Malone, K. E., Heagerty, P. J., Alexander, G. M., Gatti, L. A., Firpo, E. J., Daling, J. R., and Roberts, J. M. Expression of cell-cycle regulators p27 and cyclin E, alone and in combination, correlate with survival in young breast cancer patients, Nat Med. *3:* 222-225, 1997.

18. Donnellan, R., Kleinschmidt, I., and Chetty, R. Cyclin E immunoexpression in breast ductal carcinoma: pathologic correlations and prognostic implications., Hum Pathol. *32:* 89-94, 2001.

19. Keyomarsi, K. and Herliczek, T. The role of cyclin E in cell proliferation, development and cancer. *In:* L. Meijer, S. Guidet, and M. Philippe (eds.), Progress in Cell Cycle Research, Vol. 3, pp. 171-191. New York: Plenum Press, 1997.

20. Wang, A., Yoshimi, N., Suzui, M., Yamauchi, A., Tarao, M., and Mori, H. Different expression patterns of cyclins A, D1, and E in human colorectal cancer, J Cancer Res Clin Oncol. *122:* 122-126, 1996.

21. Scuderi, R., Palucka, K. A., Pokrovskaja, K., Bjorkholm, M., Wiman, K. G., and Pisa, P. Cyclin E overexpression in relapsed adult acute lymphoblastic leukemias of B-cell lineage, Blood. *8:* 3360-3367, 1996.